ORIGINAL ARTICLE

Isolation and in vitro susceptibility to amphotericin B, itraconazole and posaconazole of voriconazole-resistant laboratory isolates of *Aspergillus fumigatus*

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Objectives To select voriconazole-resistant mutants of *Aspergillus fumigatus* in the laboratory from drug-susceptible clinical isolates and examine their in vitro susceptibility to amphotericin B and investigational azoles, and to compare the intramycelial accumulation of voriconazole in the resistant isolates with that in the susceptible parent.

Methods Voriconazole-resistant *Aspergillus fumigatus* isolates were selected in the laboratory from three highly susceptible (MIC ≤ 0.5 mg/L) clinical isolates by stepwise selection on peptone yeast extract glucose (PYG) agar containing 0.5 mg and 4 mg voriconazole/L. Twenty-three colonies that grew in the presence of 4 mg voriconazole/L on PYG agar (frequency 1.9×10^{-8}) were tested for their in vitro susceptibility to amphotericin B, itraconazole, voriconazole and posaconazole by a broth macrodilution technique. The accumulation of voriconazole in the mycelia of two representative resistant isolates (VCZ-W42 and VCZ-W45) was determined by a previously described bioassay.

Results The geometric mean MICs (mg/L) of amphotericin B, itraconazole, voriconazole and posaconazole for these isolates were 0.45 ± 0.19, 0.69 ± 0.45, 5.24 ± 3.74 and 0.27 ± 0.18, respectively. A comparison of the geometric mean MICs of the antifungals obtained for the resistant isolates to those of the susceptible parents showed 1.15-, 2.76-, 16.90- and 1.42-fold increases, respectively, for amphotericin B, itraconazole, voriconazole and posaconazole, suggesting that low-level cross-resistance exists between the azole antifungals. The susceptible parent and the resistant isolates accumulated similar amounts of voriconazole.

Conclusions These results suggest that spontaneous mutants of *Aspergillus fumigatus* resistant to voriconazole could emerge among clinical isolates under selection pressure and that the observed reduced in vitro susceptibility to voriconazole may not be due to reduced accumulation of the drug in the mycelia.

Keywords Antifungal resistance, voriconazole, posaconazole, susceptibility studies, *Aspergillus fumigatus*

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INTRODUCTION

Voriconazole is a new investigational triazole that exhibits in vitro and in vivo activities against a wide spectrum of pathogenic yeasts [1–5], including those isolates resistant to fluconazole [6–9]. In contrast to fluconazole, which shows poor activity against pathogenic molds, voriconazole has excellent in vitro activity against a wide variety of pathogenic filamentous fungi, including rare and emerging opportunistic molds [5,10–16]. In addition to the in vitro studies, animal models showed that voriconazole is highly effective in the treatment of pulmonary aspergillosis in rabbits, guinea pigs and rats [17–19]. Moreover, there are several case reports of successful voriconazole therapy against fungal infections, including those caused by rare and newly emerging fungal pathogens [20–23], suggesting that voriconazole is a promising new azole antifungal agent for the treatment of fungal infections caused by a wide spectrum of fungal pathogens.

Being a member of the azole family of antifungal agents, voriconazole arrests fungal growth by inhibiting lanosterol 14α-demethylase [24], an enzyme involved in the synthesis of ergosterol in fungi. Although the voriconazole MICs for the fluconazole- and itraconazole-resistant fungal isolates were several-fold lower than those of fluconazole and itraconazole, our previous studies, as well as those of others, showed that low-level cross-resistance exists between fluconazole and voriconazole in *Candida* [7,9], and between itraconazole and

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voriconazole in *Aspergillus* [10]. The presence of low-level cross-resistance to voriconazole in *Candida* and *Aspergillus* species suggests that, under selection pressure, high-level resistance to voriconazole could emerge in nature. Clinical isolates of pathogenic yeasts and molds with reduced susceptibility to voriconazole could become a serious problem in the future for the treatment of fungal infections with this drug. In order to study the possible mechanisms of resistance to voriconazole in *Aspergillus* species, we selected several isolates of *A. fumigatus* with reduced susceptibility to voriconazole in the laboratory. In this paper we describe the selection, in vitro susceptibility and intracellular accumulation of voriconazole in laboratory-selected *A. fumigatus* isolates with reduced susceptibility.

MATERIALS AND METHODS

Organisms

Voriconazole-susceptible (MIC ≤ 0.5 mg/L) clinical isolates of *A. fumigatus* F55064 (ATCC 208995), W73355 (ATCC 208996) and H27023 (ATCC 208997) used in this study were obtained from the Microbiology Laboratory of the Detroit Medical Center, Michigan, USA. The original cultures obtained on Saboraud Dextrose agar slants were subcultured on the same medium to ensure purity and viability. Long-term storage of the isolates was done using conidial suspensions in 25% glycerol at $-80\,^{\circ}$C. Working cultures of the isolates were maintained on Saboraud Dextrose agar slants at $4\,^{\circ}$C.

Selection of voriconazole-resistant isolates

Voriconazole-resistant isolates were selected in the laboratory using a two-step selection process. Briefly, conidial suspensions were prepared from 6-day-old cultures and the conidial density was determined as described previously [25]. Approximately 1×10^6 conidia/plate were spread on peptone yeast extract glucose (PYG: peptone 1 g, yeast extract 1 g, glucose 3 g, agar 15 g/L of distilled water) agar plates (20 plates/organism) containing 0.5 mg voriconazole/L. *A. fumigatus* colonies that grew on PYG agar plates in the presence of 0.5 mg voriconazole/L after 3 days of incubation at $35\,^{\circ}$C (F1 colonies) were collected and stored as conidial suspensions at $-80\,^{\circ}$C.

In the second step of the selection process, conidia (1×10^6/plate) from 20 representatives of the F1 colonies which showed voriconazole MICs ≥ 1 mg/L were plated on PYG agar (20 plates/colony) containing 4 mg voriconazole/L. The agar plates were incubated in plastic sleeves for 6 days at $35\,^{\circ}$C. Colonies that grew in the presence of 4 mg voriconazole/L were collected and stored as conidial suspensions in glycerol at $-80\,^{\circ}$C. These isolates obtained after the second step of selection (F2 colonies) were used for subsequent studies.

MIC determination

The in vitro susceptibilities of various isolates of *A. fumigatus* to antifungal agents were determined by a broth macrodilution technique as previously described [25–27]. Briefly, fresh conidia were collected [28] from *A. fumigatus* isolates and suspended in RPMI 1640 at a density of 2×10^4 conidia/mL. Two times the required concentrations of the drugs were prepared in the same medium (0.5 mL) by serial dilution in sterile 6-mL polystyrene tubes (Falcon 2054, Becton Dickinson, Lincoln Park, NJ, USA) and inoculated with an equal volume (0.5 mL) of the conidial suspension. The tubes were incubated at $35\,^{\circ}$C for 48 h and scored for visible growth after vortexing the tubes gently. The MIC was defined as the lowest concentration of the drug which produced no visible growth (i.e. 100% inhibition). Each MIC determination was performed in duplicate and the experiment was repeated once. The concentrations of the antifungal agents used for the MIC studies ranged from 0.0625 mg/L to 16 mg/L. A drug-free growth control and a set of tubes with RPMI 1640 alone for monitoring contamination of the medium were used.

Determination of voriconazole accumulation in mycelia by bioassay

The intracellular accumulation of voriconazole in actively growing *A. fumigatus* mycelia was measured as follows. Fifty-milliliter cultures of various isolates of *A. fumigatus* were grown in PYG broth by inoculating the medium with 2×10^4 conidia/mL. The cultures were grown for 24 h at $35\,^{\circ}$C on a gyratory shaker at 160 rev/min. Voriconazole was added to the actively growing cultures to obtain a final concentration of 10 mg/L, and the cultures were incubated with voriconazole for 1 h. At the end of the incubation, mycelia were collected by vacuum filtration and washed rapidly two times (200 mL each) with ice-cold 10 mM Tris (pH 7.0) buffer. The washed mycelia were collected by filtration, excess water was removed by vaccum filtration, and the mycelia were immediately stored at $-80\,^{\circ}$C. The frozen mycelia ($\sim$0.25 g wet weight) were ground for 5 min in the presence of dry ice and an equal weight of acid-washed sterile sand to a fine powder using a mortar and pestle. The ground mycelial powder was suspended in 1 mL of 0.1 M sodium phosphate (pH 7.2) buffer and centrifuged at 14 000 rev/min in a microfuge. The clear supernatant was collected and sterilized by filtration through a Millipore filter (0.22 µm HWAP) attached to a 2-mL syringe. The protein contents of the mycelial extracts were determined [29] to monitor the grinding efficiency of various samples. The mycelial extracts were standardized by adjusting their protein content to 2 mg/mL using 0.1 M phosphate buffer and either used immediately or stored at $-80\,^{\circ}$C until further use.

The concentrations of voriconazole in the mycelial extracts were determined by a plate inhibition assay similar to that described by Martin et al [18]. Briefly, PYG agar plates

containing 100 mL medium/plate were prepared in large Petri dishes (150 mm × 15 mm; Falcon, Becton Dickinson). An overnight culture of *Candida kefyr* was grown in PYG broth and the culture was diluted with the same medium to a cell density of 1×10^6 cells/mL. One-milliliter aliquots of this cell suspension were spread on PYG agar, and the plates were incubated at 35 °C for 3 h to absorb the excess liquid. Circular holes 5 mm in diameter were cut into the agar using a sterile cork borer, and 0.1-mL aliquots of the samples containing voriconazole were placed in the wells. The plates were incubated at 35 °C overnight, and the zone of inhibition around the circular well was measured. For the construction of a standard curve, the zones of inhibition for several concentrations (0.625–10 mg/L) of voriconazole were determined. The standard curve was constructed by plotting the concentration of voriconazole against the diameter of zone of inhibition.

To study the direct effect of mycelial extract on the antifungal activity of voriconazole, we incubated 1 μg of voriconazole with 0.1 mL of mycelial extract (2 mg protein/mL) at 35 °C for 2 h. The antifungal activity of the mycelial extract-treated voriconazole was determined by the plate inhibition assay, and the results were compared with the activity of voriconazole untreated with the mycelial extract.

Antifungal agents

Itraconazole (R51 211, batch no. STAN-9304-005-1) was obtained from Janssen Pharmaceutica, Beerse, Belgium. Voriconazole was obtained from Pfizer Pharmaceuticals, New York, NY, USA. Amphotericin B (batch no. 20-914-29670) was obtained from the Squibb Institute for Medical Research, Princeton, NJ, USA. Posaconazole (SCH56592) was obtained from Schering-Plough Research Institute, Kenilsworth, NJ, USA. All the antifungals were dissolved in dimethylsulfoxide at a concentration of 1 mg/mL and stored as 0.25-mL aliquots at −20 °C. The frozen stock was thawed at room temperature and gently vortexed several times to ensure that any remaining crystals were completely dissolved before use. Where applicable, comparable concentrations of dimethylsulfoxide were used to examine its effect on the growth of the organism.

RESULTS

Selection of resistant mutants

Our attempts to select *A. fumigatus* isolates with reduced in vitro susceptibility to voriconazole using a single-step selection in the presence of ≥4 mg voriconazole/L on PYG agar failed to yield any isolate with reduced susceptibility to voriconazole. We therefore used a two-step selection process for the isolation of resistant mutants. Conidia prepared from the highly susceptible *A. fumigatus* F55064, W73355 and H27023 were screened initially on PYG agar containing 0.5 mg voriconazole/L. A large number of colonies were obtained (frequency 1.56×10^{-4}) after the initial selection. These colonies were designated the F1 generation.

Conidial suspensions were prepared from 60 selected representatives of the F1 colonies whose voriconazole MICs were ≥1 mg/L, and screened in the presence of 4 mg voriconazole/L on PYG agar. Twenty-three colonies grew from 1.2×10^9 conidia after 6 days of incubation at 35 °C with a frequency of 1.9×10^{-8}. These colonies were subcultured in the absence of the antifungal agent and tested for their in vitro susceptibility to voriconazole with a broth macrodilution technique. All colonies showed reduced susceptibility to voriconazole after several passages on drug-free medium. The genetic stability of two representative resistant isolates (VCZ-W42 and VCZ-W45) was determined by repeated passage in the absence of selection pressure. Even after 20 such passages in the absence of voriconazole, these mutant isolates showed reduced susceptibility to voriconazole. Working cultures of these mutants were routinely maintained on PYG agar slants in the absence of voriconazole, or as conidial suspensions at −80 °C.

In vitro susceptibility study

The in vitro susceptibilities of resistant *A. fumigatus* isolates selected from the parental strains F55064 ($N = 9$), W73355 ($N = 12$) and H27023 ($N = 2$) on 4 mg voriconazole/L to various antifungal agents are shown in Table 1. All isolates had significantly higher voriconazole MICs compared to those

Table 1 A comparison of the in vitro susceptibilities of laboratory-selected voriconazole-resistant isolates of *A. fumigatus* to various anitifungal agents with those of the parental strains and of clinical isolates

	MICs (mg/L; geometric mean ± standard deviation) of antifungals[a]			
Aspergillus fumigatus	**AMB[b]**	**ITZ[b]**	**VCZ[b]**	**PCZ[b]**
Parental strains ($N = 3$)	0.39 ± 0.14	0.25 ± 0.0	0.31 ±0.14	0.19 ± 0.07
VCZ-resistant isolates ($N = 23$)	0.45 ± 0.19	0.69 ± 0.45	5.24 ± 3.74	0.27 ± 0.18
Clinical isolates ($N = 284$)	0.34 ± 0.21	0.42 ± 0.56	0.27 ± 0.21	0.17 ± 0.11

[a]Data obtained for a typical experiment. The experiment was repeated once and the MICs obtained for the same isolates were within ±1 dilution. [b]AMB, amphotericin B; ITZ, itraconazole; VCZ, voriconazole; PCZ, posaconazole.

obtained for the susceptible parents (11–24-fold increase) and the clinical isolates (20–23-fold increase). The geometric mean (GM) MIC value of voriconazole for the resistant isolates was 5.24 ± 3.74 mg/L. No significant rise in amphotericin B MIC was obtained (GM $= 0.45 \pm 0.19$ mg/L), whereas a ~2–3-fold increase in the MIC of itraconazole was obtained (GM $= 0.69 \pm 0.45$ mg/L) compared to those of the susceptible parents (GM $= 0.25 \pm 0.0$ mg/L) and the clinical isolates (GM $= 0.42 \pm 0.56$ mg/L). On the other hand, only a marginal rise in MIC (~1.42-fold) was noted for posaconazole (GM $= 0.27 \pm 0.18$ mg/L) compared to those of the susceptible parents and the clinical isolates. Two representative isolates (VCZ-W42 and VCZ-W45) from the voriconazole-resistant group were selected for further studies.

Plate inhibition assay

Since radioactive voriconazole with high specific activity suitable for uptake studies is not readily available at present, we used a sensitive bioassay for the determination of intracellular voriconazole in the mycelia. *Candida kefyr* is highly susceptible to voriconazole, and in the presence of low concentrations of the drug the growth of the organism is almost completely suppressed and a distinct zone of inhibition is obtained, as the drug readily diffuses into the media in a circular fashion from a point source, forming a concentration gradient. The clear zone of inhibition obtained for *Candida kefyr* in the presence of voriconazole is in sharp contrast to those of other *Candida* species (e.g. *Candida albicans*), which produce a hazy or translucent zone of inhibition. The diameter of the zone of inhibi-

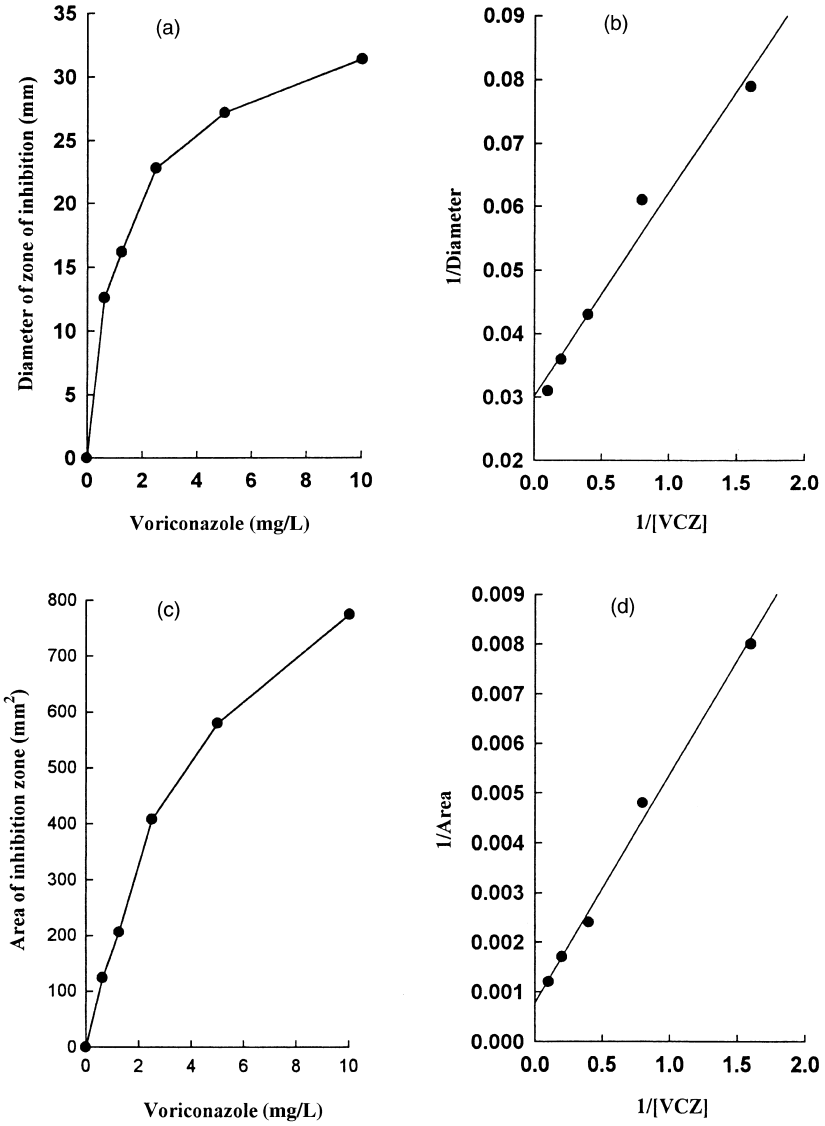

Figure 1 Dose response of plate inhibition assay of voriconazole: (a) diameter of zone of inhibition versus voriconazole concentration; (b) 1/Diameter versus 1/[voriconazole]; (c) area of inhibition zone versus voriconazole concentration; (d) 1/Area versus 1/[voriconazole]. The curves in (b) and (d) were fitted by linear regression using SigmaPlot 3.0, Jandel Scientific Software, San Rafael, CA, USA.

tion is proportional to the concentration of the drug applied at the point source. As shown in Figure 1a, a plot of the diameter of the zone of inhibition against known concentrations of the drug (standard curve) produces a hyperbola. The approximate concentration of an unknown sample can be read off from the standard curve by intrapolation. Alternatively, one can plot the reciprocal of the concentration of the drug (1/[voriconazole]) against the reciprocal of the diameter of the zone of inhibition (1/Diameter), which produces a linear relationship. From this linear double reciprocal plot, the concentration of an unknown sample can be estimated more accurately by intrapolation (Figure 1b).

Further analysis of the data revealed that plotting the area of the zone of inhibition (instead of the diameter of the zone of inhibition) against concentration of the drug produces a linear relationship at lower concentrations (Figure 1c). A more accurate calculation of the concentration of the unknown sample will be obtained from this curve by intrapolation. Moreover, as shown in Figure 1d, a double reciprocal plot provides a better linear fit, and a more accurate value of the unknown sample will be obtained by intrapolation. Using this technique, we estimated the accumulation of voriconazole in the resistant isolates and compared the value with that obtained for the susceptible parent.

Effect of *A. fumigatus* mycelial extract on the antifungal activity of voriconazole

The sensitivity and the reproducibility of the bioassay for the detection of intracellular voriconazole in the mycelial extracts depend on the extent of interaction of the drug with the components of the mycelial extracts. Therefore, the primary objective of this experiment was to examine whether *A. fumigatus* mycelial extract has any direct effect on the antifungal activity of voriconazole. This is particularly important in the plate inhibition assay, where mycelial extract could either inactivate voriconazole, or sequester it, making the drug unable to exert its antifungal activity. Thus, any observed reduction in the activity of voriconazole could be an artifact rather than due to a reduction of the intracellular accumulation of the drug. As shown in Table 2, various amounts of voriconazole (1.25 mg/L to 10 mg/L) incubated in the presence and absence of the mycelial extract showed almost identical activities, suggesting that *A. fumigatus* mycelial extract has no effect on the antifungal activity of voriconazole. In addition to the mycelial extract prepared from the voriconazole-susceptible isolate W73355, we tested the effect of the mycelial extracts from two voriconazole-resistant isolates on the activity of voriconazole. As shown in Table 2, mycelial extracts prepared from either *A. fumigatus* VCZ-W42 or *A. fumigatus* VCZ-W45 had no effect on the anti-*Candida kefyr* activity of the drug.

Table 2 Effect of mycelial extracts prepared from voriconazole-susceptible and -resistant *A. fumigatus* on the antifungal activity of voriconazole as determined by plate inhibition assay[a]

Source of mycelial extract	Concentration of voriconazole (mg/L) treated with mycelial extract	Inhibition of *Candida kefyr* growth (diameter of inhibition zone in mm)	
		Without mycelial extract treatment[b]	With mycelial extract treatment[c]
A. fumigatus W73355	1.25	17.0 ± 0.0	17.0 ± 0.0
	2.5	23.0 ± 0.0	21.5 ± 0.70
	5	27.0 ± 0.0	26.5 ± 0.70
	10	31.5 ± 0.70	31.5 ± 0.70
A. fumigatus VCZ-W42	10	34.0 ± 1.0	34.7 ± 1.15
A. fumigatus VCZ-W45	10	33.8 ± 0.5	34.3 ± 1.52

[a]See Materials and methods for details; [b]0.1 mL of phosphate buffer was added to the reaction mix instead of mycelial extract; [c]0.1-mL aliquots of the mycelial extract (2 mg protein/mL) prepared from the indicated *A. fumigatus* isolate were used.

Accumulation of voriconazole in resistant isolates

Reduced susceptibility to antibiotics due to diminished accumulation of the drug within the cell has been described in a wide variety of organisms, ranging from bacteria to mammalian cells, and including fungi [30–33]. The diminished intracellular accumulation of the antifungal agent in fungi could be due to reduced penetration of the drug across the permeability barrier, defective drug uptake mechanisms (in cases where the antifungal is taken up by a transport system), or efflux pumps, where the drug accumulated intracellularly is pumped out at the expense of energy. We therefore investigated the intracellular levels of voriconazole in the resistant mutants and compared the results with that of the susceptible parent to examine the possible reduction of voriconazole accumulation in resistant isolates. The susceptible parent and the resistant mutants accumulated almost identical amounts of the drug when mycelia were incubated with voriconazole for 1 h (Figure 2). A known concentration (1 mg/L) of voriconazole used as a reference produced an almost identical zone of inhibition to that produced by the mycelial extracts. This suggests that approximately 1 mg/L of voriconazole was present in the mycelial extracts. This value represents approximately 0.2% of the total amount of voriconazole (500 μg) used in the uptake study. These data suggest that it is unlikely that decreased accumulation of the drug in the mycelia is the primary reason for the reduced susceptibility to voriconazole in these resistant isolates.

DISCUSSION

The voriconazole-resistant isolates of *A. fumigatus* used in our study were selected in the laboratory from the same parental strains. These are spontaneous variants that may have possessed identical genetic alteration(s). Thus, it is possible that the multiple isolates we studied may be clones of the same isolate, since they all underwent the same selection process. On the other hand, it is highly unlikely that all the F2 generation colonies that we obtained from three different parental strains underwent identical spontaneous random genetic changes resulting in identical isolates that showed reduced susceptibility to voriconazole. A detailed study of the mechanism(s) associated with the resistance in several isolates is required to understand whether they all possess the same mechanism for voriconazole resistance.

The cytochrome P450-dependent lanosterol demethylase ($P450_{LDM}$) is known to be the cellular target of action of azoles. The azole antifungal agents mimic the natural substrate lanosterol and bind to the enzyme, preventing access of the natural substrate to the active site [34]. Since all azoles are known to exert their antifungal activity by inhibiting $P450_{LDM}$, one would expect cross-resistance to members of the azole family of antifungal agents among resistant isolates. Our results on the susceptibility of voriconazole-resistant isolates to itraconazole and posaconazole showed low-level (modest rise in MIC) cross-resistance. The occurrence of low-level cross-resistance to

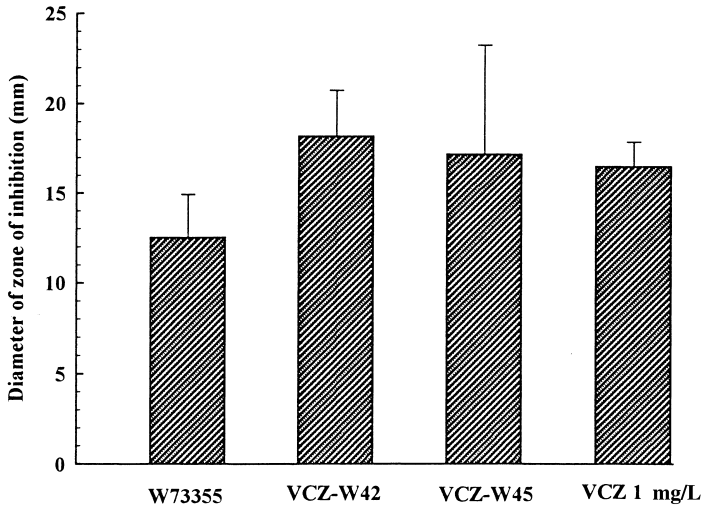

Figure 2 Accumulation of voriconazole in resistant and susceptible *A. fumigatus* isolates. Each histogram represents the mean of two independent experiments, and in each experiment the bioassay was done in triplicate. The standard deviation of the sample is shown above each histogram. Pairwise comparisons of the mean values by two-tailed *t*-test showed no significant difference between the susceptible parent (W73355) and the resistant isolates (VCZ-W42 and VCZ-W45). A known concentration (1 mg/L) of voriconazole was used as a reference.

azoles among resistant isolates was previously found in other fungi as well. For instance, fluconazole-resistant *Candida albicans* [4,7,9] and *Cryptococcus neoformans* [8] showed only low-level resistance to itraconazole and voriconazole. The exact reason(s) for such variable susceptibility to members of the same class of compounds among resistant isolates is not understood. It probably has to do with the binding affinities of various azoles for the active site of the enzyme.

As more and more investigational drugs are being developed as antifungal agents whose radioisotopic forms are not readily available, sensitive, reproducible bioassay represents an alternative to the use of radiolabeled molecules for uptake studies. The main drawback of using a bioassay is its poor sensitivity compared to the use of radioisotopes. However, in the case of voriconazole, even a fraction of 1 μg of the drug could be detected by the plate inhibition assay, due to the high sensitivity of *Candida kefyr* to voriconazole. Another factor in the successful application of the bioassay for the detection of voriconazole is its lack of interaction with the mycelial extract. If the drug interacts with the mycelial extract, then release of the intracellularly accumulated drug by homogenizing the mycelia would either inactivate the drug or sequester it, and in both cases the drug would be unable to exert its antifungal activity. Although there are drawbacks in the use of bioassays to detect antifungal agents, our experiments with voriconazole suggest that bioassays could be used for the detection of azoles accumulated in *A. fumigatus* mycelia.

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